REMARKS

The specification has been amended to indicate that the instant application is a 371 national stage filing of PCT/GB00/03573.

Claim 26 has been canceled. Claim 1 has been amended. Support for this amendment can be found in Applicant's specification, especially at page 2 and in Table 2 on page 20. New claim 42 has been added. No new matter has been added by way of this amendment.

After entry of this amendment claims 1, 2 and 42 will be pending.

Rejections under 35 U.S.C. § 112

Indefiniteness

Claim 26 was rejected under 35 U.S.C. § 112, second paragraph as being indefinite. Claim 26 has been canceled making this rejection moot.

Enablement

Claims 1, 2 and 26 were rejected under 35 U.S.C. § 112, first paragraph as allegedly not enabling any person skilled in the art to make and use the invention commensurate in scope with the claims. Claim 26 has been canceled making the rejection to this claim moot.

Amended claim 1 is drawn to a GST having at least 90% sequence identity to SEQ ID NO: 10 and having a 50-fold increase in specific activity toward fomesafen in the presence of homoglutathione than in the presence of glutathione.

The Office Action states that the specification does not support the broad scope of the claims because it does not provide guidance for which amino acids of SEQ ID NO: 10 can be altered and to which other amino acids, and which amino acids must not be changed, to maintain

GST activity. The Action concludes that because of the unpredictability of the art and lack of guidance in the specification, undue experimentation would have been required by one skilled in the art to develop and evaluate amino acid sequences which have 80% identity to SEQ ID NO: 10.

In the enablement context, the question of undue experimentation is a matter of degree. The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation must not be unduly extensive. The application must enable one of ordinary skill in the art to practice the full scope of the claimed invention. That does not mean that the specification itself must necessarily describe how to make and use every possible variant of the claimed invention, for the artisan's knowledge of the prior art and routine experimentation can often fill gaps, interpolate between embodiments, and perhaps even extrapolate beyond the disclosed embodiments, depending upon the predictability of the art. *Chiron Corp. v. Genentech, Inc.*, 363 F.3d 1247.

It is respectfully submitted that the present invention as amended can be practiced without undue experimentation. Although molecular evolution is arguably an unpredictable art, knowledge of which regions of the protein may or may not be changed is not necessary when identifying which GST proteins having 90% identity to SEQ ID NO: 10 are capable of providing at least a 50-fold increase in activity toward fmesafen in the presence of homoglutathione than in the presence of glutathione. It is routine for the skilled person to perform sequence alignments in order to determine percent identity. Therefore, the instant specification provides ample direction and guidence for the skilled person to identify a GST having at least 90% sequence identity to SEQ ID NO: 10 (*See* Example 1). The instant specification also gives the skilled person guidence as to how to determine which such GSTs provide at least a 50-fold increase in specific activity toward fomesafen in the presence of homoglutathione than in the presence of glutathione (*See* Example 4, and Table 2).

Thus, Applicants respectfully submit that the enabling disclosure of the instant specification is commensurate in scope with the claims as amended and request that the rejections under 35 U.S.C. § 112, first paragraph be withdrawn.

Rejection under 35 U.S.C. § 102(e)

Claim 26 was rejected under 35 U.S.C. §102(e) as being anticipated by McGonigle *et al.* (US Patent 6,063,570). Claim 26 has been canceled making the rejection to this claim moot.

Rejection under 35 U.S.C. § 103(a)

Claims 1, 2 and 26 were rejected as being unpatentable over McGonigle *et al.* (US Patent 6,063,570) in view of Gulick *et al.* (1995 Proc. Natl. Acad. ci. 92: 8140-8144) and Skipsey *et al.* (1997 FEBS Letters 409:370-374).

This rejection under § 103 is based on the contention that McGonigle *et al.* discloses a soybean GST having 84% identity to SEQ ID NO: 10 and teaches the usefulness of this sequence for the detoxification of herbicides. The Examiner suggests that it would have been obvious to the skilled person to evolve the GST taught by McGonigle *et al.* using methods taught by Gulick *et al.* to select for GSTs with increased activity against commonly used soybean herbicides. Applicants respectfully disagree.

While McGonigle *et al.* teaches a soybean GST having 84% identity to SEQ ID NO: 10 and teaches the usefulness of this GST for the detoxification of herbicides, it does not teach or suggest the usefuleness of a GST having 90% identity to SEQ ID NO: 10 for the detoxification of any diphenylether type herbicide, particularly fomesafen. Given that the skilled person would know that the substrate specificity toward any particular GST would be quite variable, there is no motivation for the skilled person to select the GST disclosed by McGonigle *et al.* simply because it exhibited the closest identity as the starting point for molecular evolution. In fact, it is respectfully submitted that the skilled person would actually select, as a starting point, a GST that had at least been shown to be active toward diphenylether herbicides, such as fomesafen. The test for obviousness is surely not whether the skilled person could use the GST disclosed by McGonigle *et al.*, but whether the skilled person would use the GST disclosed by McGonigle *et al.* given the motivation in the prior

art. Applicants respectfully submit that there is no motivation for the skilled person to select the GST taught by McGonigle *et al.* in the absence of the teaching of the present invention. Even if the skilled person were arguably motivated to evolve the GST taught by McGonigle *et al.*, there is absolutely no expectation of success in obtaining a GST with 90% identity to SEQ ID NO: 10 which exhibits at least a 50-fold increase in activity toward fomesafen in the presence of homoglutathione rather than glutathione as the present invention requires.

The Examiner asserts that Gulick *et al.* teaches methods of evolving a GST encoding sequence to create an enzyme which more efficiently detoxifies a particular compound of interest which comprises mutating selected regions of the GST substrate binding site followed by selection for mutants having increased resistence to the compound of interest.

Applicants attach herewith a reference (Dixon *et al.* 2003 J. Biol. Sci. 278: 23930-23935) published after the filing date of the instant application, which Applicants believe is the first publication of an example of forced evolution of a plant GST toward a herbicide substrate. Dixon *et al.* describe the evolution of maize GSTs toward the diphenylether herbicide, fluorodifen. The results demonstrate that out of a screen of 5,000 evolved mutants, only 7 were isolated that exhibited significantly higher GST activity towrd fluorodifen (*See* Figure 1), with the best performing mutant exhibiting 19-fold increse toward fluorodifen. This was in contrast to the results obtained with fomesafen and acifluorfen, two closely related diphenylether herbicides. Thus, Dixion *et al.* teaches that the evolution (as suggested by Gulick *et al.*) of plant GSTs toward herbicide substrates, in particular fomesafen, is not a routine art. Applicants respectfully submit that the skilled person would not arrive at the present solution (i.e. a GST having 90% identity with SEQ ID NO: 10 which has at least a 50-fold increase in activity toward fomesafen in the presence of homoglutathione than in the presence of glutathione) as a consequence of evolution experiments suggested by Gulick *et al.* either alone or in combination with McGonigle *et al.*

The Examiner asserts that Skipsey *et al.* teach that a number of selective soybean herbicides, including acifluorfen and fomesafen, are detoxified by conjugation with homoglutathione.

Applicants respectfully submit Skipsey *et al.* merely suggests that a GST with high activity toward fomsafen is a desirable objective. The deficiencies of McGonigle *et al.* and Gulick *et al.*

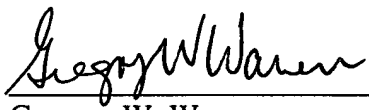
have been highlighted above. Skipsey *et al.* fails to remedy these deficiencies. Thus, the solution provided by the present invention cannot be considered obvious in light of Skipsey *et al.* alone or in combination with Gulick *et al.* and McGonigle *et al.* Hence, the references, considered alone or in combination fial to render the claimed invention obvious.

Reconsideration and withdrawl of the rejection under 35 U.S.C. § 103(a) is therefore respectfully requested.

CONCLUSION

Pursuant to the foregoing remarks, Applicants respectfully submit that all of the pending claims fully comply with 35 U.S.C. § 112 and are allowable over the prior art of record. No new matter is added by way of this amendment. Reconsideration of the application and allowance of all pending claims is earnestly solicited. Should the Examiner wish to discuss any of the above in greater detail; or deem that further amendments should be made to improve the form of the claims, then the Examiner is invited to telephone the undersigned at the Examiner's convenience.

Respectfully submitted,



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Forced Evolution of a Herbicide Detoxifying Glutathione Transferase*[S]

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Plant Tau class glutathione transferases (GSTUs) detoxify diphenylether herbicides such as fluorodifen, determining their selectivity in crops and weeds. Using reconstructive PCR, a series of mutant GSTUs were generated from *in vitro* recombination and mutagenesis of the maize sequences *Zm*GSTU1 and *Zm*GSTU2 (with the prefix *Zm* designating *Zea mays* L.). A screen of 5000 mutant GSTUs identified seven enzymes with enhanced fluorodifen detoxifying activity. The best performing enhanced fluorodifen detoxifying mutant (EFD) had activity 19-fold higher than the parent enzymes, with a single point mutation conferring this enhancement. Further mutagenesis of this residue generated an EFD with a 29-fold higher catalytic efficiency toward fluorodifen as compared with the parents but with unaltered catalysis toward other substrates. When expressed in *Arabidopsis thaliana*, the optimized EFD, but not the parent enzymes, conferred enhanced tolerance to fluorodifen. Molecular modeling predicts that the serendipitous mutation giving the improvement in detoxification is due to the removal of an unfavorable interaction together with the introduction of a favorable change in conformation of residues 107–119, which contribute to herbicide binding.

The relative rate of herbicide detoxification in crops and weeds is a primary determinant of their selectivity (1). Crops rapidly metabolize herbicides by oxidative or hydrolytic reactions followed by conjugation with sugars or peptides and vacuolar sequestration of the polar products (1). In weeds, these detoxification reactions are slower. Glutathione *S*-transferases (GSTs)[1] have a well characterized role in determining the metabolism and selectivity of chloroacetanilide, thiocarbamate, and chloro-*s*-triazine herbicides in maize (2). GSTs catalyze the conjugation of these herbicides with the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) to form non-toxic *S*-glutathionylated products (2). In other crops, alternative GST activities determine herbicide selectivity. For example, photobleaching diphenylether herbicides such as fluorodifen are rapidly detoxified by GSTs in legumes (see Fig. 1*A*) but less efficiently in maize (3). Species-dependent GST-mediated detoxification can be explained by differences in the expression of the six distinct families of plant GST genes, classified as the Phi, Zeta, Tau, Theta, Lambda, and dehydroascorbate reductase classes (4–6). The plant-specific Phi and Tau GSTs are primarily responsible for herbicide detoxification, showing class specificity in substrate preference. Phi enzymes (GSTFs) are highly active toward chloroacetanilide and thiocarbamate herbicides, whereas the Tau enzymes (GSTUs) are efficient in detoxifying diphenylethers and aryloxyphenoxypropionates (7, 8). In maize, GSTFs are the major class of expressed GST (7), whereas in soybean, GSTUs predominate, with this difference accounting for the differential detoxification of different classes of herbicide in the two crops (4).

We have been interested in enhancing the detoxifying potential of plant GSTs using the dual approach of DNA shuffling and directed mutagenesis. In maize (*Zea mays* L.), *Zm*GSTU1 and *Zm*GSTU2 are the major Tau enzymes expressed, although considerably less abundant than the *Zm*GSTFs (9, 10). With the intention of enhancing their detoxifying activity toward fluorodifen, we have used reconstructive error-prone PCR (11) to randomly generate mutant GSTUs derived from both *Zm*GSTU1 and *Zm*GSTU2. After large scale screening of the mutants from this screen, the most improved GST was then subjected to selective mutagenesis to further increase activity. The resulting optimized catalysts were then used to transform *Arabidopsis thaliana* and tested for their ability to increase herbicide tolerance *in planta*.

EXPERIMENTAL PROCEDURES

Generation of Mutant GSTs—Clones pGSTU1 and pGSTU2, pBluescript SK$^-$ vectors containing cDNA sequences for *Zm*GSTU1 and *Zm*GSTU2, respectively, were obtained from cDNA expression library antibody screens as detailed previously (9, 10). In the work describing their original isolation, *Zm*GSTU1 was termed *Zm*GSTV and *Zm*GSTU2 was termed *Zm*GSTVI, the new designations reflecting a unifying change in nomenclature (5). For reconstructive PCR, 10 μg of each of the plasmids pGSTU1 and pGSTU2 was digested with DNase I (0.25–2.0 units) in 50 μl of 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$ at 22 °C for 10 min with the reaction stopped with 2 μl of 0.5 M EDTA. Reactions producing fragments of mainly 0–500 bp were used for reconstructive PCR (11). Using 20 ng/μl digested DNA, after 50 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s + 3 s/cycle), reaction products of 500 bp–20 kb were obtained, and 1 μl of this mixture was used as template for a standard 50-μl PCR reaction using M13 forward and reverse oligonucleotide primers (30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s). PCR products of the expected size were recloned into pBluescript.

Screening Mutants for GST Activity—Wells of sterile flat-bottomed, 96-well microtiter plates, each containing 200 μl of LB medium supple-

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The atomic coordinates and structure factors (code 1OYJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplementary data providing sequences for EFD mutant clones pEFD1–7.

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[1] The abbreviations used are: GST, glutathione *S*-transferase; GSTF, Phi class GST; GSTU, Tau class GST; CDNB, 1-chloro-2,4-dinitrobenzene; EFD, enhanced fluorodifen detoxifying mutant; *Zm*, from *Z. mays* L.

mented with antibiotics, were inoculated with individual mutant bacterial colonies taken from a master plate and grown for 16 h to stationary phase. The plates were centrifuged, and the growth medium was removed by aspiration prior to resuspending the bacteria in 200 μl of 0.1 M glycine-NaOH, pH 9.5, containing 0.5 mM fluorodifen and 5 mM glutathione. Plates were incubated at 37 °C (2–4 h), and the release of *p*-nitrophenol was visually monitored. Mutants showing at least a 5-fold faster accumulation of the yellow *p*-nitrophenol than the parent *Zm*GSTU clones were selected for further characterization.

Analysis of Recombinant Mutant GSTs—GSTUs cloned in either pBluescript or pET plasmids in *Escherichia coli* were cultured without isopropyl-1-thio-β-D-galactopyranoside induction, and the respective recombinant proteins were purified using *S*-hexylglutathione-Sepharose (9, 10). Purified GSTs were extensively dialyzed to remove *S*-hexylglutathione and assayed for activity toward CDNB (1-chloro-2,4-dinitrobenzene) and fluorodifen (12), the latter by following the increase in absorbance at 400 nm after incubation at 30 °C in 0.1 M glycine-NaOH buffer, pH 9.5, containing 5 mM glutathione and 50 μM fluorodifen. High pressure liquid chromatography-based assays for activity toward herbicides other than fluorodifen were as described (13) except that assays with fomesafen were carried out in glycine-NaOH buffer, pH 9.5. The protein concentration of purified recombinant GSTs was measured based on their calculated UV absorbance at 280 nm (14).

Site-directed Mutagenesis—The Q115L point mutation in EFD6 (enhanced fluorodifen detoxifying mutant 6), corresponding to the 113th amino acid residue of *Zm*GSTU2, was subjected to PCR using specific mutagenic primers, together with a primer to the T7 promoter, using pEFD6 as the template. PCR products were subcloned back into pEFD6 to give constructs expressing the mutants EFD6–115E, EFD6–115N, EFD6–115F, EFD6–115A, and EFD6–115Q containing mutations of leucine 115 to glutamic acid, asparagine, phenylalanine, alanine, and glutamine, respectively.

Modeling of Fluorodifen Binding to ZmGSTU2—The *Zm*GSTU2 protein was modeled on the templates of two Tau class enzymes, wheat *Ta*GSTU4 (8) and rice *Os*GSTU1 (Protein Data Bank accession code 1OYJ), showing 39 and 63% sequence identity to *Zm*GSTU2, respectively, using the web-based utility SWISSMODEL (www.expasy.ch/swissmod/SWISS-MODEL.html). The model obtained was manually analyzed using the graphics program QUANTA (Accelyrs Inc.) for steric clashes and chemical sensibility. The conformation of the side chain of residue Gln-115 resulted in a steric clash with Tyr-170 and therefore was remodeled using water positions from the *Os*GSTU1 as a guide. A molecular model of fluorodifen was generated using INSIGHTII and CATALYST (Accelyrs Inc.), and a suitable low energy conformation was docked into the active site using QUANTA.

Generation and Analysis of Transgenic Plants—GST sequences were subcloned into pRT108 by PCR from the respective pET-vectors and ligated into the *Nco*I and *Bam*HI sites of the vector (15). This cassette was then digested with *Hind*III and ligated into similarly digested pCAMBIA 3300 (CAMBIA, Canberra, Australia). The resulting vectors were transformed into *Agrobacterium tumefaciens* strain C58C3 and used to transform *A. thaliana* ecotype Columbia by floral dipping (16). Dipped plants were grown to seed set, and T1 plants were grown for 10 days in the greenhouse (16-h photoperiod; 23 °C day; 18 °C night) in a 4:1 mixture of general purpose compost and silver sand prior to spraying with 0.02% (w/v) glufosinate ammonium (1 ml/35 cm^2) to select for transformants. Surviving plants were resprayed after 7 days. T1 lines showing good GST expression were selected for propagation after analyzing extracts from individual rosette leaves by SDS-PAGE and Western blotting using an anti-*Zm*GSTU1–2 serum (9). For each line of interest, ~10 whole T2 seedlings (14 days old) were extracted and assayed for GST activity and immunoreactive polypeptides by Western blotting after normalizing for total protein content. For fluorodifen spray trials, cells containing five 25-day-old T2 seedlings grown in compost, selected previously for the presence of T-DNA insertions by spraying with glufosinate ammonium to kill segregating wild-type plants, were sprayed with 20 μM fluorodifen formulated in 1% (v/v) acetone and 0.01% (v/v) Tween 20 daily for 14 days with each 23-cm^2 cell receiving a 1-ml application.

RESULTS

Generation and Characterization of Mutant GSTUs by Reconstructive PCR—*Zm*GSTU1 and *Zm*GSTU2, showing 72% sequence identity to one another (9, 10), were employed for reconstructive PCR using equal amounts of DNA fragments of pGSTU2 (pBluescript containing cDNA encoding *Zm*GSTU2) and pGSTU1 (pBluescript containing cDNA encoding *Zm*GSTU1). Following *in vitro* recombination, a single product was amplified by PCR from each reassembled template and cloned into pBluescript SK^-. A microtiter plate screening procedure was used to assay 5000 mutant colonies for enhanced GST activity toward the herbicide fluorodifen based on the release of colored *p*-nitrophenol (Fig. 1, *A* and *B*). As compared with the parent *Zm*GSTUs, a significant proportion of mutants showed moderately enhanced activity, largely due to relatively higher levels of GST expression in the bacteria. Analysis of three such clones showed that they were chimeras of the N-terminal portion of *Zm*GSTU1 with the remaining sequence composed of *Zm*GSTU2. In each case, the specific activity of the pure enzyme toward fluorodifen was little affected. However, seven mutants were identified that had at least 5-fold higher enzyme activity (as judged visually) than bacteria expressing either *Zm*GSTU1 or *Zm*GSTU2. These EFD mutant clones pEFD1–7, expressing the mutant proteins EFD1–7, were sequenced (see supplementary information). Six of the seven EFDs were highly expressed, containing N termini (between 48 and 62 residues) derived from *Zm*GSTU1 (Fig. 1*C*). With the exception of EFD6, the remainder of the coding sequence was derived from *Zm*GSTU2, interrupted by a region, or regions, of varying size derived from *Zm*GSTU1 (Fig. 1*C*). In EFD6, there was no *Zm*GSTU1 insertion in the C-terminal half of the protein, but

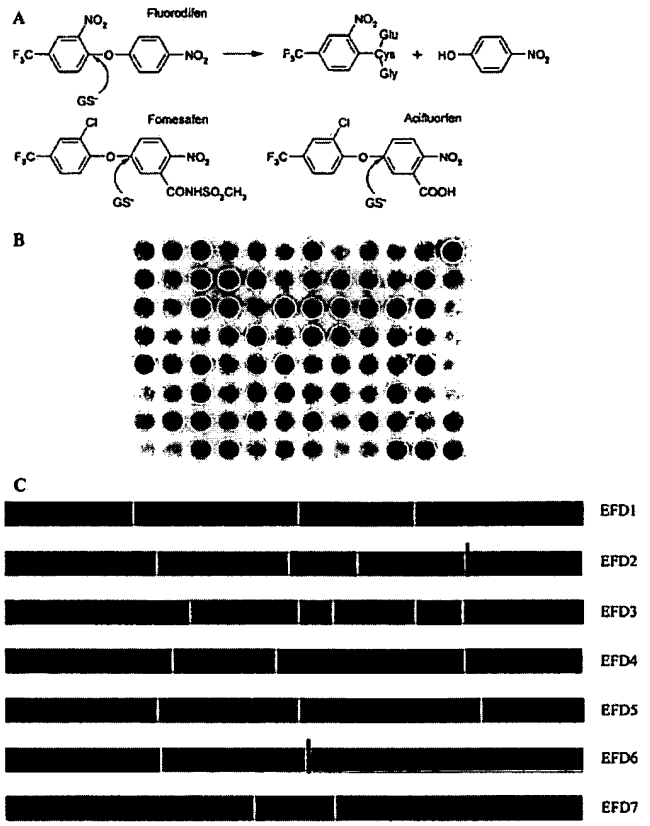

FIG. 1. **The generation and screening of EFD mutants showing an increased glutathione-dependent detoxification of fluorodifen to *p*-nitrophenol and *S*-(2-nitro-4-trifluoromethylphenyl)-glutathione.** Also shown are the other diphenylether herbicides tested, fomesafen and acifluorfen, with the sites of glutathione attack indicated (*A*). The *E. coli* colonies expressing the mutant GSTUs were incubated with fluorodifen and glutathione in microtiter plates, and the release of *p*-nitrophenol was visually assessed (*B*). From a screen of 5000 mutants, seven EFDs were isolated that showed significantly higher GST activity toward fluorodifen, and on sequencing, these mutants were shown to be derived from a combination of recombination of the parent *Zm*GSTU1 (*black*) and *Zm*GSTU2 (*gray*) sequences and point mutations (*vertical bars*) (*C*).

TABLE I

Kinetic characteristics of purified mutated and wild-type recombinant GSTUs toward the diphenylethers fluorodifen (Flu), acifluorfen (Aci), and Fomesafen (Fom) and the model spectrophotometric substrate CDNB, as well as GSH

ND, no detectable activity.

Enzyme	k_{cat}/K_m (Flu)	k_{cat}/K_m (Aci)	k_{cat}/K_m (Fom)	k_{cat}/K_m (CDNB)	K_m (CDNB)[a]	V_{max} (CDNB)[a]	K_m (GSH)[b]
	$M^{-1}\cdot S^{-1}$	$M^{-1}\cdot S^{-1}$	$M^{-1}\cdot S^{-1}$	$M^{-1}\cdot S^{-1}$	μM	*nanokatal/mg*	*mM*
*Zm*GSTU1	230	1.63	6.70	18,400	1010	1110	0.56
*Zm*GSTU2	400	ND	0.42	300,000	115	1910	1.72
EFD1	5290	0.77	0.98	219,000	206	2290	1.95
EFD2	3700	1.10	1.26	142,000	136	930	0.76
EFD3	7550	0.96	0.84	343,000	197	1950	1.15
EFD4	2360	0.62	1.50	185,000	204	1730	1.18
EFD5	2500	0.68	1.70	144,000	212	1500	1.05
EFD6	4290	7.60	9.10	9200	1800	760	4.75
EFD6–115Q	220	ND	0.52	193,000	106	880	0.92
EFD6–115F	5040	4.20	3.80	47,400	534	870	0.74
EFD6–115A	11,700	4.90	2.60	150,000	200	1060	0.78

[a] Apparent values, calculated at 5 mM glutathione.
[b] Apparent values, calculated at 1 mM CDNB.

instead a single missense mutation corresponding to the substitution of glutamine with leucine at residue 115 (equivalent to residue 113 of *Zm*GSTU2) was identified.

The seven EFD mutants were individually subcloned into the pET-11d plasmid, resulting in the expression of a 28-kDa polypeptide in each case as determined by SDS-PAGE. The kinetics of each EFD was then examined after purifying the enzymes by affinity chromatography, with the exception of EFD7, which was not analyzed further due to the instability of its activity. The recombinant GSTs were assayed with fluorodifen and the related diphenylether herbicides fomesafen and acifluorfen (Fig. 1*A*). All diphenylether substrates were used at relatively low concentrations (50 μM) due to their limited solubility in water. This made it impossible to directly determine the K_m values toward these substrates, which were well above the solubility limit in each case. Instead, a value for enzyme catalytic efficiency (k_{cat}/K_m) was calculated in each case (Table I). Of the diphenylethers tested, both parent GSTUs and related EFDs showed a marked substrate preference for fluorodifen. The k_{cat}/K_m values for fluorodifen were higher in all the EFDs relative to the parent enzymes. This was in contrast to the results obtained with fomesafen and acifluorfen, with the mutant enzymes EFD1–5 showing similar or slightly reduced values for k_{cat}/K_m relative to *Zm*GSTU1, the only parent showing appreciable activity toward both herbicides. EFD6 did show a modest 4-fold increase in k_{cat}/K_m toward acifluorfen relative to *Zm*GSTU1 as compared with a greater than 18-fold enhancement with fluorodifen as substrate. These results suggested that gene shuffling studies had generated a set of mutant enzymes that had selectively improved activities toward fluorodifen but not toward closely related substrate chemistries. Constrained by their limited solubilities, it was not possible to further investigate the effects of gene shuffling on enzyme binding and turnover of the diphenylethers using kinetic analysis. However, it was possible to determine the effect of mutagenesis on substrate binding and turnover of the model substrate CDNB. Mutants EFD1–5 inclusive had apparent K_m values toward GSH and CDNB and associated V_{max} values that were within the range determined for the parent GSTUs. This was what would be predicted from mutant enzymes that are effectively chimeras of modular blocks of sequence from the two parents. In contrast, EFD6 had increased K_m values toward both CDNB and GSH, coupled with a decreased V_{max}. Taken together with the singular changes seen with EFD6 in the cleavage of fluorodifen, these results collectively suggested that this mutant had undergone a more radical change in active site topography than the other EFDs. With the exception of the substitution of residue 115, EFD6 was otherwise a simple chimera of the N-terminal portion of *Zm*GSTU1 and the remaining sequence of *Zm*GSTU2 (Fig. 1*C*). Based on the essentially conserved kinetic characteristics of the other chimeric EFDs in which blocks of sequence had been shuffled, it was concluded that the substitution at residue 115 was a key factor determining the novel detoxifying activities of EFD6. Attention was therefore focused on the selective mutagenesis of this residue.

Directed Mutagenesis of EFD6—A side effect of the L115Q substitution in EFD6 was that the mutant enzyme was predominantly expressed in the insoluble fraction in *E. coli*, unlike the other chimeric EFDs analyzed. Again, this was consistent with this substitution having a major effect on protein structure. Rational site-directed mutagenesis was employed to substitute the mutated residue and determine the effect on enzyme activity with the aim of finding a mutant with both high activity toward fluorodifen and more stable expression as a soluble protein. The original mutation replaced glutamine, a medium-sized neutral residue, with leucine, a smaller hydrophobic residue. This residue was therefore mutated to asparagine (neutral but smaller), glutamic acid (acidic, same size), phenylalanine (hydrophobic, large), or alanine (hydrophobic, small), giving the mutants EFD6–115N, EFD6–115E, EFD6–115F, and EFD6–115A, respectively. Residue 115 was also mutagenized back to glutamine to yield EFD6–115Q, effectively producing the unmutated chimera of *Zm*GSTU1 and *Zm*GSTU2. Each of these mutants was expressed in *E. coli* as a LacZ fusion protein. Lysates from bacteria expressing EFD6–115E or EFD6–115N had negligible GST activity with the misfolded recombinant polypeptides precipitated in the inclusion bodies. In contrast, bacteria expressing EFD6–115F, EFD6–115A, and EFD6–115Q gave good yields of soluble GSTs, which were subjected to kinetic analysis (Table I). EFD6–115Q had characteristics similar to *Zm*GSTU2 toward both diphenylethers and CDNB, confirming that the point mutation in EFD6 affecting residue 115 was primarily responsible for the enhanced fluorodifen cleaving activity. As determined by comparing k_{cat}/K_m values, mutation of residue 115 to phenylalanine to produce EFD6–115F gave an enzyme with similar activity toward fluorodifen, reduced activities toward fomesafen and acifluorfen, and a 5-fold increase with CDNB as substrate. The latter change was effected by decreasing the K_m toward CDNB rather than increasing V_{max}. The most successful attempt at directed mutagenesis was seen with the substitution of residue 115 of EFD6 to alanine. The resulting mutant EFD6–115A had almost a 3-fold increase in k_{cat}/K_m toward fluorodifen relative to EFD6. Overall, the combination of DNA shuffling and directed mutagenesis of key residue 115 had

caused a 29-fold increase in detoxifying activity toward fluorodifen relative to the most active parent enzyme *Zm*GSTU2. As compared with EFD6, the alanine substitution also restored a greater k_{cat}/K_m value toward CDNB, again as a result of lowering K_m toward the substrate, giving an enzyme with essentially similar kinetic characteristics toward CDNB and glutathione to the parent *Zm*GSTUs, and suggesting that this mutant did not have the structural problems of EFD6. Similarly, the "optimized" mutant enzyme showed only minor differences in k_{cat}/K_m toward acifluorfen and fomesafen, herbicides resembling fluorodifen. When the structurally unrelated chloroacetanilide herbicides acetochlor, alachlor, and metolachlor were assayed as GST substrates, EFD6–115A showed specific activities of 112 picokatal/mg of pure protein, 72 picokatal/mg, and 144 picokatal/mg, respectively. These values were very similar to those determined with the purified *Zm*GSTUs assayed under identical conditions (*Zm*GSTU1, 160 picokatal/mg of pure protein 68 picokatal/mg, and 192 picokatal/mg, respectively; *Zm*GSTU2, 215 picokatal/mg of pure protein, 91 picokatal/mg, and 157 picokatal/mg, respectively).

Effect of Expression of EFD Mutants on Tolerance to Fluorodifen in Planta—Transgenic *Arabidopsis* plants were individually engineered to express the parent *Zm*GSTUs, EFD6, EFD3, and EFD6–115A under the control of the CaMV35S promoter. This combination of EFDs was selected as EFD6–115A represented the optimized detoxifying enzyme generated through a combination of gene shuffling and directed mutagenesis, whereas EFD3 was the most efficient fluorodifen detoxifying enzyme generated by shuffling alone. None of the resulting T1 or T2 transgenics showed any abnormal phenotype under normal growth conditions. T2 plants were then analyzed for the expression of the introduced GSTUs by Western blotting using an antiserum raised to the *Zm*GSTU1–2 heterodimer and by assaying for fluorodifen cleaving activity (Fig. 2*A*). The antiserum recognized 28-kDa polypeptides in all GSTU-transformed lines except those expressing *Zm*GSTU2, which was due to the weak immunoreactivity of this protein with this antibody (9). When assayed for fluorodifen detoxifying activity (Fig. 2*A*), the only lines showing enhanced GST activity toward the herbicide in *in vitro* assays were those transformed with EFD3 and EFD6–115A, the latter showing 19-fold greater specific activity than the controls. It was then of interest to determine whether or not the transgenic expression of the herbicide detoxifying GST activity could confer tolerance to fluorodifen *in planta*.

Fluorodifen was originally developed as a selective photobleaching herbicide for use in crops of peanuts, soybean cotton, and rice rather than in the more sensitive *Brassica* species (17). The selectivity of fluorodifen is associated with its greater rate of detoxification in tolerant crops as compared with susceptible weeds, principally through GST-mediated metabolism (3, 17). It would therefore be anticipated that enhanced expression of a detoxifying GST in a normally susceptible species would give visibly greater tolerance to the photobleaching activity of fluorodifen, which is a characteristic feature of diphenylether herbicides even in tolerant crops (13). *Arabidopsis* plants were found to be highly sensitive to fluorodifen when sprayed as an unformulated leaf drench treatment. Careful adjustment of the treatments showed that a repeated spraying of 25-day-old *Arabidopsis* plants with 20 μM fluorodifen gave a sublethal but severe photobleaching, which largely arrested growth. T2 generation controls and plants expressing *Zm*GSTU1, *Zm*GSTU2, or the optimized mutant EFD6–115A were then treated with the fluorodifen dosing regime, and the plants were visually assessed. After a 14-day treatment, the vector control, *Zm*GSTU1 and *Zm*GSTU2-expressing plants, were severely stunted

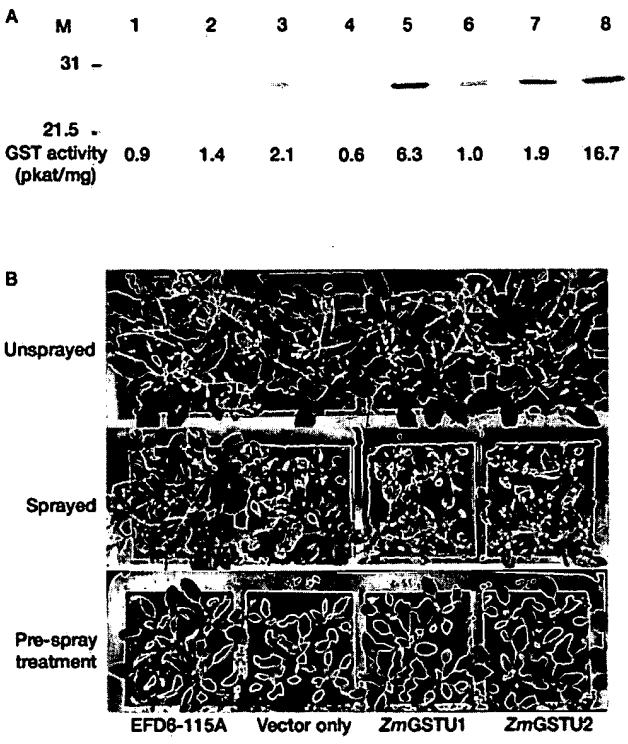

FIG. 2. **Analysis of T2 transgenic Arabidopsis.** As shown in *A*, pools of plants derived from each construct were analyzed by SDS-PAGE and Western blotting using an anti-*Zm*GSTU1–2 serum. *Lane M*, molecular mass markers (kDa); *1*, wild type; *2*, vector control (pCAMBIA3300); *3*, *Zm*GSTU1; *4*, *Zm*GSTU2; *5*, EFD3; *6*, EFD6 (line 1); *7*, EFD6 (line 2); *8*, EFD6–115A. The GST activities toward fluorodifen determined in duplicate crude extracts are shown as mean values (picokatal/mg total protein). As shown in *B*, plants transformed with vector alone, *Zm*GSTU1, *Zm*GSTU2, or EFD6–115A were treated daily with 20 μM fluorodifen (*sprayed*) or were left untreated (*unsprayed*) for 14 days in a glasshouse.

with the foliage showing signs of photobleaching and necrosis (Fig. 2*B*). Similarly treated plants expressing EFD6–115A were much less damaged, and although showing some photobleaching, had continued to grow at a rate similar to that of unsprayed plants.

The recent availability of crystal structures of GSTUs from wheat (8) and rice (Protein Data Bank accession number 1OYJ) has permitted molecular modeling of *Zm*GSTU2 as well as the EFD mutants binding to fluorodifen. EFD1–5 are essentially *Zm*GSTU2 with two regions of *Zm*GSTU1 in the N-terminal domain and C-terminal domain, respectively (Fig. 3*A*). These changes in the N-terminal region were correlated with high levels of GST expression and a lower K_m toward glutathione as compared with the other parent *Zm*GSTU1 (Table I).

DISCUSSION

Through a combination of random and directed mutagenesis, we have derived a GST, EFD-115A, with a greatly increased capacity to detoxify fluorodifen and demonstrated that this mutant enzyme can give enhanced protection against the photobleaching injury incurred by this herbicide when expressed *in planta*. Although forced evolution directed toward pesticide-degrading enzymes derived from microorganisms has been proposed in engineering herbicide tolerance (18), to our knowledge, this is the first report of a plant enzyme engineered for such an application. Mammalian GSTs have been the subject of several attempts to engineer their detoxifying activities using both gene shuffling and directed evolution with various degrees of success (19). Using random mutagenesis selectively directed to the active site domains responsible for drug binding, a rat

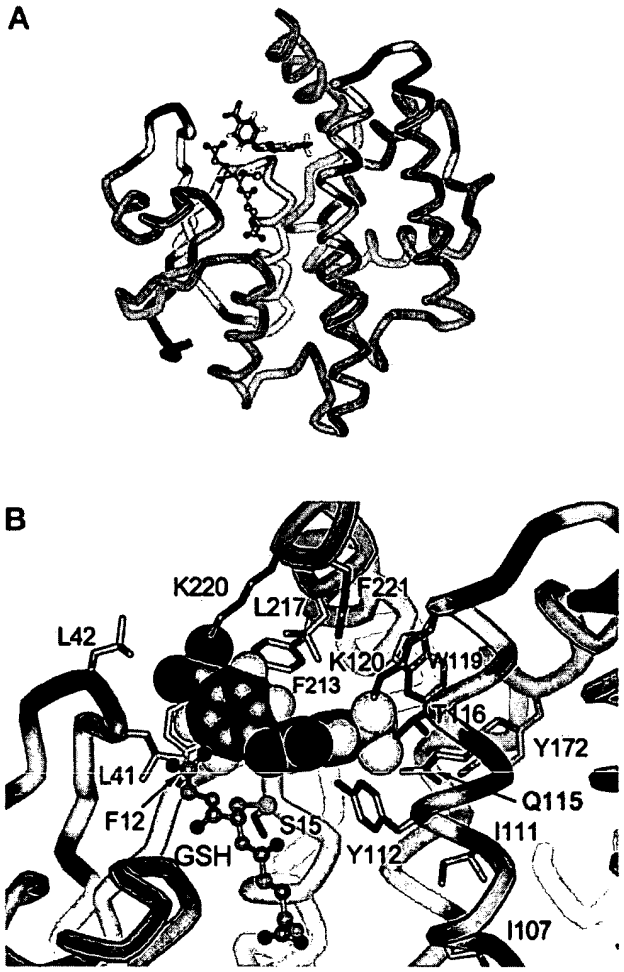

FIG. 3. *A*, a ribbon representation of the structural model of *Zm*GSTU2 monomer colored to highlight the changes in a representative EFD mutant enzyme (EFD2). The region derived from *Zm*GSTU2 is colored *green*, that from *Zm*GSTU1 is colored *light blue* with regions that differ from *Zm*GSTU2 highlighted in *dark blue*, and the critical residue 115 is highlighted in *red*. Glutathione is shown in ball-and-stick representation, and the docked herbicide fluorodifen is represented in stick form, with both colored according to atom type. *B*, a detail of the active site of the *Zm*GSTU2 model with fluorodifen represented in Van der Waals spheres. Residues predicted to be important for fluorodifen binding are shown in stick form and colored according to atom type.

GST was engineered such that it conferred a 30-fold increase in the tolerance of *E. coli* to the alkylating toxin merchlorethamine when expressed in the bacteria (20). When analyzed, the mutant rat GST showed a 6-fold increase in catalytic efficiency in detoxifying merchlorethamine but a major decrease in CDNB conjugation as compared with the parent enzyme. Cassette mutagenesis has also been used to increase the activity of human GSTA1-1 toward CDNB, with the mutants screened for their binding to transition state analogues of reaction intermediates using phage display (21, 22). As found with the EFDs, whereas chimeras of related mammalian GSTs gave activities that were hybrids of the activities of the parents (23), greater success in engineering novel activities have been through combining DNA shuffling with mutagenesis (24).

In the approach we have adopted, through the process of random recombination and mutagenesis and subsequent directed substitution of an unanticipated key residue at 115, we have evolved a GST whose activity toward fluorodifen is greatly and selectively enhanced. Surprisingly, the affinity and turnover of the enzyme toward other even closely related xenobiotic substrates was largely unaffected. A more common experience of forced evolution of GSTs would be that the enhancement of one activity is at the expense of another (20). In fact, the switching in catalytic efficiency toward different GST substrates following mutagenesis has been demonstrated in rational design studies based on protein structural information (19). The re-engineering of the active site of human GSTA1-1 to support massively improved conjugation of alkenals resulted in a 20-fold decrease in activity toward CDNB (25). In the absence of precise structural information on EFD6–115A and the parent *Zm*GSTUs, it is difficult to categorically define the basis of this selective fine-tuning in catalytic efficiency toward fluorodifen. However, following the conclusion of the mutagenesis program, the structures of GSTUs from wheat (8) and rice (Protein Data Bank accession number 1OYJ) were solved, permitting the molecular modeling of the parent *Zm*GSTU2 as well as the series of EFD mutants. This structural information has helped explain some of the changes in substrate specificity and catalysis seen in the generation of the EFD series. Firstly, the EFD1–5 are essentially *Zm*GSTU2 with blocks of interceding sequences from *Zm*GSTU1 in the N-terminal domain involved in glutathione binding and the C-terminal domain involved in herbicide binding (8). These chimeras were each associated with high levels of recombinant protein expression in *E. coli*. Mutants EFD2–5 had K_m values toward GSH that were lower than that determined in *Zm*GSTU2, whereas EFD1 showed a similar affinity for the thiol. Comparisons of the sequences of the EFDs around the glutathione binding domain suggested the substitutions of L42G, D43N, and G45S in EFD2–5, which were not present in EFD1, directly influenced binding affinity for the thiol co-substrate. With respect to the differences in binding of the hydrophobic co-substrates seen in these mutants, it is known that the first helix of the C-terminal domain forms an important structural determinant of binding (8, 26, 27). Comparisons of the variations of the sequences of chimeras EFD1–5 within this helix identifies only four differences in this region between *Zm*GSTU1 and *Zm*GSTU2 that can account for differences in substrate binding; one surface-exposed S114C faces away from the active site, whereas I107V, I111L, and Q115G are all on the same face of the helix, buried in the hydrophobic core of the protein. The other differences are surface-exposed and too far from the H-site to be significant. Although these sequence/activity comparisons for EFD1–5 help to explain relatively modest changes in enzyme activity, the serendipitous discovery that residue 115 was important provided a specific impetus for defining a role for this residue in catalysis. Molecular modeling predicts that Gln-115 will be orientated such that the amide oxygen would be in close proximity to the trifluromethyl group of fluorodifen, which would be unfavorable for binding (Fig. 3*B*). The absence of this interaction alone does not confer high fluorodifen activity, as demonstrated by the low activity in the parent *Zm*GSTU1. Clearly positive interactions from other residues in *Zm*GSTU2 are also important. The single point mutation Q115L would lead to the burying of the leucine within the first helix of the C-terminal domain and introduce a potential steric clash with Y172. We predict that a rearrangement of this part of the helix leads to an indirect effect on binding and turnover, residue 115 being otherwise too far removed from the active site to interact with the substrate. Modeling of the mutant EFD6 predicts that the disruption of the helix would lead to the side chain of Lys-109 moving closer to residue Glu-70 (of the dimer-related molecule), which is known to be involved in glutathione binding (8). The potential formation of a salt bridge between Lys-109 and Glu-70 could then explain a weaker binding of glutathione, witnessed by the 5-fold increase in K_m toward GSH observed with EFD6 (Table I). In *Zm*GSTU2, the residues involved in

binding fluorodifen within the first helix are Tyr-112, Thr-116, Trp-119, and Lys-120 with Tyr-112 co-coordinating the nitro group of the herbicide (Fig. 3*B*). Residues Leu-217, Lys-220, and Phe-221 from the very C-terminal helix of the enzyme are also potentially important in herbicide binding with Lys-220 predicted to interact with the NO_2 of the second phenyl ring. Since none of these residues involved in herbicide binding is substituted in the EFDs, we conclude that the increased fluorodifen detoxifying activity is due to the removal of an unfavorable interaction by the amide of Gln-115 together with the introduction of a favorable change in conformation of residues 107–119, which contribute to the hydrophobic binding site.

The forced evolution of xenobiotic metabolizing enzymes in plants has great potential in manipulating the detoxification of toxins, agrochemicals, and pollutants (18). The process of such engineering also provides useful insights into the evolution of metabolism-based resistance to pesticides and pollutants. In the case of herbicide resistance in weeds, in the few cases studied in detail, detoxification-based resistance is associated with the increased expression of existing detoxifying enzymes including GSTs (28). However, in view of the diversity in species and populations in which herbicide resistance has arisen, it is likely that as with the EFDs, enhanced metabolism due to recombination and mutagenesis of key detoxifying enzymes has made its own unique contribution to this growing problem.

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